



#4 1623
NIDN-10431

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: A. Cuthbertson Group Art Unit: 1623
Serial Number: 10/052,300 Examiner: To be assigned
Filing Date: January 18, 2002
Title: Process for the Deprotection of Protected Thiols

Completion of Claim for Priority

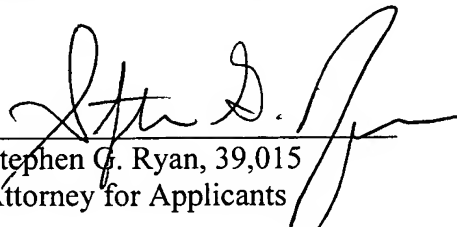
Assistant Commissioner for Patents
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Applicants hereby submit the official certified copy of the priority document number **GB 9916919.5**, **GB 0003926.3**, and **GB 0007865.9** in connection with the above identified application, benefit of which is claimed in the declaration of this application. The Examiner is most respectfully requested to acknowledge receipt of this certified copy in the next Official Office Action.

Respectfully submitted,


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P. Mahoney

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P01/7700 0.00 9916919.5

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19 JUL 1999

The Patent Office
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1. Your reference 8.3.70306

2. Patent application number
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3. Full name, address and postcode of the
or of each applicant (underline all surnames)

Nycomed Imaging AS
Nycoveien 2
P.O. Box 4220 Torshov
N-0401 Oslo
Norway

Patents ADP number (if you know it)

If the applicant is a corporate body, give
country/state of incorporation

Norway

6266961001

4. Title of the invention

Process for the deprotection of
protected thiols

5. Name of your agent (if you have one)

Frank B. Dehn & Co.

"Address for service" in the United Kingdom
to which all correspondence should be sent
(including the postcode)

179 Queen Victoria Street
London
EC4V 4EL

Patents ADP number (if you know it)

166001

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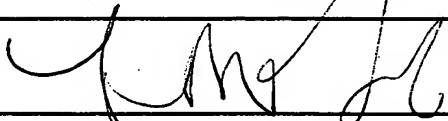
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Signature Date 19 July 1999

12. Name and daytime telephone number of person to contact in the United Kingdom
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Process for the deprotection of protected thiols

5 This invention relates to a new process for the deprotection of protected thiol compounds, more particularly thiols protected by acetamidomethyl (from hereon referred to as Acm) groups, as well as to oxidation of the deprotected thiols to form disulphides.
10 Such processes are particularly useful in peptide synthesis.

 During organic syntheses it is quite routine for certain reactive functionalities to be protected to prevent their participation in unwanted side reactions.
15 For example, reactive carbonyl functionalities are often protected as ketals, and reactive hydroxyl and carboxyl groups are often protected as esters.

 The neutral but strongly nucleophilic thiol group present in cysteine generally requires protection during peptide syntheses. A wide variety of thiol protecting groups are known, including benzyl, 4-methylbenzyl, 4-methoxybenzyl, trityl, methoxytrityl, t-butyl, t-butylthiol, acetyl, 3-nitro-2-pyridinesulphenyl and Acm.
20 All these groups have been successfully used in peptide synthesis and are reviewed by Barany and Merrifield in "The Peptides" Vol. 2, Ed. Gross and Minehoffer, Academic Press, pp. 233-240 (1980).
25

 Acm is a thiol protecting group which is normally removed by oxidative cleavage, for example by treatment with mercury (II), iodine, silver (I) or thallium (III).
30 It is generally regarded as acid stable since, although acidolytic cleavage of Acm is theoretically possible in anhydrous or aqueous acids, such reactions are inconveniently slow in practice because of difficulties in protonating the sulphur atom.
35

 In this context Fujii et al. in Chem. Pharm. Bull. 41(6), pp. 1030-1034 (1993) describe the synthesis and

oxidation of oxytocin using Cys(Acm) and trifluoroacetic acid (TFA)/10% dimethyl sulphoxide (DMSO). The authors state that Cys(Acm)-oxytocin survived nearly intact after a 12 hour treatment in the above TFA/DMSO mixture, showing that Acm protection is stable under such acid conditions. The S-Acm group was also reported by Veber et al. in J. Am. Chem. Soc. 94, pp. 5456-5461 (1972) as being stable to hydrofluoric acid (HF) and strong nucleophiles such as hydrazine.

Van Rietschoten et al. reported in Peptides (1977), pp. 522-524 that treatment of a peptide containing four Acm groups with HF-anisole resulted in 20% of the Acm groups being removed. More recently, Fisher et al. in J. Pep. Res. 49(4), pp. 341-346 (1997) have described a modification to a tyrosine residue due to acidolytic cleavage of Acm, and Singh et al. in Tetrahedron Letters Vol. 37, No. 24, pp. 4117-4120 (1996) report on the partial acidolytic cleavage of Acm from C-terminal Cys(Acm) peptides. These acid-induced deprotection reactions are regarded as unwanted side reactions during peptide cleavage.

The present invention is based on the unexpected finding that Acm thiol protecting groups become increasingly labile to acids at temperatures in excess of 30°C, such that it is possible to achieve substantially quantitative deprotection with reaction times of one hour or less, e.g. as little as 10 minutes. Such acid-induced deprotection is particularly advantageous in that it avoids the need for use of the more toxic reagents currently employed to remove Acm groups. By conducting the deprotection in the presence of an appropriate oxidising agent, liberated thiol groups may be converted directly to disulphide groups; as discussed hereinafter this has particularly valuable applications in the synthesis of cyclic peptides containing disulphide linkages.

Thus, according to one aspect, the invention

provides a process for the deprotection of an acetoamidomethyl-protected thiol which comprises reacting said protected thiol with an acid at a temperature above 30°C, advantageously at or above 50°C.

5 Both aqueous and anhydrous acids may be used in the deprotection process. Thus, for example, aqueous inorganic acids, e.g. mineral acids such as hydrochloric acid, and aqueous or anhydrous organic acids, e.g. carboxylic acids such as acetic acid or, more
10 preferably, strong carboxylic acids such as TFA, and sulphonic acids such as methanesulphonic acid may be useful.

DMSO is a preferred example of an oxidising agent useful in converting liberated thiol groups to
15 disulphide groups.

In a preferred embodiment the thiol to be deprotected is a peptide containing one or more Ac-m-protected cysteine residues.

Peptides represent a class of molecules which are
20 extremely well suited for the targeting of disease specific markers *in vivo*, and considerable attention is being given to the preparation of synthetic peptides as potential components of targeted imaging agents.

The synthesis of cysteine-containing peptides
25 presents special challenges to a peptide chemist as the peptide can exist in either a reduced or an oxidised state. Oxidised peptides containing more than one cysteine residue may form intramolecular disulphides or intermolecular disulphides such as dimers, trimers or
30 multimers. Thus, for example, a peptide containing six cysteine residues is potentially capable of forming 15 disulphide isomers, and careful planning and selection of suitable protection strategy is therefore required if correct disulphide pairings are to be achieved in such
35 peptides. It will be appreciated that correct pairing is frequently critical to correct folding of the peptide backbone and concomitant orientation of side chain

functionalities in order to give a biologically active conformation capable of high affinity receptor binding.

Typical existing strategies for the selective formation of two or more disulphide bonds use combinations of protecting groups such as trityl and Acm or t-butylthio and Acm, the first disulphide bond being formed after removal of the trityl or t-butylthio groups and the second being formed by oxidative cleavage of the Acm groups using, for example, iodine or thallium trifluoroacetate. Other examples of the synthesis of multibridged peptides include the solution synthesis of insulin by Sieber et al. described in *Helv. Chim. Acta.* 57, pp. 2617-2621 (1974) and the procedures of Atherton et al., *J. Chem. Soc. Perkin Trans. 1*, p. 2065 (1985) and Akaji et al., *J. Am. Chem. Soc.* 115, p. 11384 (1993).

Acm deprotection in accordance with the present invention permits considerable simplification of such strategies, such that two or more disulphide bonds may be generated in a "one pot reaction", thereby avoiding the need for intermediate purification of partially oxidised or partially protected peptides and so achieving savings in solvent use and time and improvements in product yield. Thus, by preparing a peptide containing two acid-labile thiol protecting groups as well as two or more Acm groups, a first disulphide bond may be formed by acid and oxidative treatment of the peptide at a relatively low (e.g. ambient) temperature and one or more further disulphide bonds may be formed simply by increasing the temperature of the reaction mixture to a temperature in excess of 30°C such that the Acm groups are cleaved.

The positions of the acid-labile protecting groups are advantageously such that the first-formed disulphide bond brings the molecule into a folded conformation such that the remaining Acm-protected thiol groups are juxtaposed in a manner which facilitates correct

formation of the remaining disulphide bond or bonds.

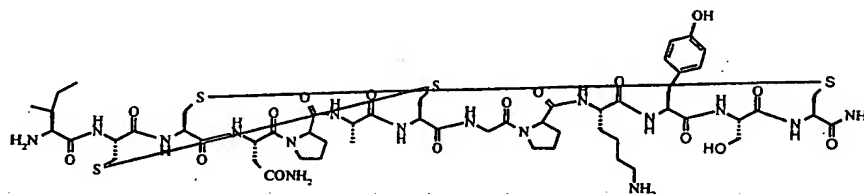
5 The use of TFA/DMSO mixtures, e.g. with a DMSO content of 10-20%, to promote deprotection and disulphide bond formation is particularly preferred in this embodiment of the invention, since both the S-protected starting materials and the disulphide linked intermediates and end products will typically be soluble in such mixtures. Both TFA and DMSO may readily be recycled for further use.

10 The present procedure allows cysteine-containing peptides to be oxidised at concentrations in excess of 1 mg/ml, thereby substantially reducing solvent volume requirements compared to existing protocols such as iodine cleavage of AcM and air oxidation, which
15 typically employ peptide concentrations of the order of 0.1 mg/ml and so require the product to be concentrated, e.g. by ion exchange chromatography, prior to final purification. In accordance with the present procedure, on the other hand, product concentration may be effected
20 simply by solvent evaporation *in vacuo*.

The following non-limitative Examples serve to illustrate the invention.

Example 1: 'One pot' oxidation of α -conotoxin SI

Ile-Cys-Cys-Asn-Pro-Ala-Cys-Gly-Pro-Lys-Tyr-Ser-Cys-NH₂
disulphides connecting Cys 2 with Cys 7 and Cys 3 with
Cys 13.



The peptide sequence was assembled on an ABI 433A automatic peptide synthesiser starting with Rink amide resin (Novabiochem) on a 0.12 mmol scale using 1 mmol amino acid cartridges. The Trityl protecting group was chosen for cysteine residues 2 and 7 while 3 and 13 were protected with acetamidomethyl groups. All amino acids were pre-activated using *O*-Benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU).

The simultaneous removal of peptide and side-chain protecting groups (except AcM) from the resin was carried out in trifluoroacetic acid (TFA) containing 5% triisopropylsilane and 5% water for 2 hours giving a crude product yield of 130 mg. HPLC analysis of crude product (Vydac 218TP54 column) was carried out using a gradient of 5 to 30 % B over 20 min (A=0.1% TFA/water and B=0.1% TFA/acetonitrile) at a flow rate of 1 mL/min. The product was found to be >90% pure. Further product characterisation was carried out using MALDI mass spectrometry; expected for AcM protected product, M+H at 1496, found, at 1502.

Procedure 1: To 2 mg of crude product was added TFA (10 mL) and dimethyl sulfoxide (DMSO) (0.1 mL). The mixture was stirred on ice and the oxidation followed by HPLC. The starting product, retention time 16.2 min (0 to 30%

B over 20 min where A=0.1% TFA/water and B=0.1% TFA/acetonitrile) was slowly replaced by a new product at 16.4 minutes. After 2h the starting product had completely disappeared. To the peptide solution was then
5 added 0.05 mL of anisole and the mixture warmed to 60°C for a further 2h following which the TFA was removed in vacuo and the peptide precipitated by the addition of diethyl ether. The crude fully oxidised product 1.4 mg comprised 2 main products; ratio 1:5, with an HPLC
10 retention time of 16.9 and 17.8 minutes respectively. The 17.8 minute product, comprising ca. 80% of the material was found to co-elute with an authentic sample of α -conotoxin (Bachem, H-1112).

The pure conotoxin was obtained by purification on
15 a Vydac 218TP152010 semi-prep column using a gradient of 0 to 30% B over 30 min (A=0.1% TFA/water and B=0.1% TFA/acetonitrile) at a flow rate of 5 mL/min. The product following freeze-drying (0.7 mg, 35% yield) was found to be >90% pure. Further characterisation was
20 carried out using MALDI mass spectrometry; expected for product, M+H at 1354, found, at 1356.

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